Clinical Chemistry

Simultaneous Quantification of Apolipoprotein A-I and Apolipoprotein B by Liquid Chromatography-Multiple Reaction Monitoring/Mass Spectrometry

Bob Barrett: This is the podcast from *'Clinical Chemistry'*. I am Bob Barrett. In order to measure concentrations of proteins in blood or serum, most clinical laboratories utilize immunoassays. However, these techniques can be affected by interfering antibodies, poor antibody specificity, cross-reactivity, and high dose hook effects. Thus, there is considerable interest in using Liquid Chromatography Multiple Reaction Monitoring Mass Spectrometry as an alternative to measure these protein biomarkers.

However, formal validation of multiplex LC/MRM-MS assays and comparisons with existing clinical immunoassays for known biomarkers have not yet been performed. In an article published recently in '*Clinical Chemistry*', Dr. Andrew Hoofnagle, a medical director of the clinical laboratories at the University of Washington in Seattle, and his team have developed a method to utilize LC/MRM-MS to quantify two important Apolipoproteins in a manner that compares well with clinically validated immunoassays.

The potential application to large clinical studies could simplify efforts to reduce the limitations that may affect immunoassays. Dr. Hoofnagle is our guest in this podcast.

Doctor, mass spectrometry has been used for a long time in the clinical laboratory to measure small molecules like drugs and hormones, why did your group decide to try to measure proteins in patient blood samples using mass spectrometry?

Dr. Andrew Hoofnagle: The standard of care for small molecules in drugs has certainly turned towards mass spectrometry for its specificity and sensitivity. Immunoassays for a long time have been recognized to not react specifically with the analytes that we are interested in, but instead have some cross-reactivity with related molecules in patient samples.

The same can be said for protein immunoassays and it's been known for a long time, but there was never a replacement platform available. With the advent of mass spectrometry, many of the advances that have been made in micro flow fluidics have became possible to actually measure proteins potentially quantitatively by mass spectrometry to replace the immunoassays and all of the problems that we have with patient samples in the clinical laboratory.

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Bob Barrett: What type of problems come up with this method and how do those problems affect the care of your patients?

Dr. Andrew Hoofnagle: The biggest problems with immunoassays in the clinical laboratory include interferences from endogenous immunoglobulins or antibodies in the patient sample. So for instance, some patients have autoantibodies directed towards the protein that we are trying to measure. Because immunoassays rely on the ability to bind the surface of those proteins, endogenous immunoglobulins or antibodies can mask that part of the molecule from binding the reagent antibody.

Those surface features are called epitopes. So autoantibodies can mask the epitopes from immunoassays and give us a falsely negative result in sandwich immunoassays.

In addition, and what's even more frightening are what are sometimes called heterophilic antibodies; what other people call anti-reagent antibodies. These are antibodies that can actually cause false positive results, especially in cancer monitoring and treatment. Patients who have been treated for cancer, we expect to have a completely negative result. However, these heterophilic or anti-reagent antibodies can bridge the gap and cause a false positive.

This has led to, for instance, with false positive data hCG or human Chorionic Gonadotropin. Those falsely positive levels have resulted in the removal of women's uterus of child bearing age. It has resulted in men who have been treated for prostate cancer, having falsely elevated PSA or prostate-specific antigen. Those false positive values resulted in further unnecessary therapy for patients and those are just two examples. There are many in the literature of inappropriate treatment, inappropriate diagnoses, and the results are severe.

So in addition to autoantibodies and these anti-reagent antibodies, there are other problems with immunoassays. For example, false negatives due to something called the high dose hook effect, which is a saturation of the reagent antibodies not allowing sandwiches to be formed, again causing false negative results and potentially missed opportunities for a proper therapy.

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The lack of specificity is also a concern, though less of a concern than it is with small molecules. The final problem with protein immunoassays is a lack of standardization between laboratories with immunoassays. The great example here in Seattle is it's possible to go to one hospital, have your TSH measured and be diagnosed hypothyroid and then go across the street and have your TSH measured in another laboratory and be diagnosed as normal.

So in one hospital, you would actually be treated for hypothyroidism and in the other hospital, you would be diagnosed as completely normal. So the immunoassays have given us problems for protein in a clinical laboratory.

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Bob Barrett: Has there been much work done on this before your group started their experiments?

Dr. Andrew Hoofnagle: Indeed. Trying to measure proteins by mass spectrometry, the field has been alive for about ten years. Most of the experiments have been directed more in shotgun experiments, which are, experiments using mass spectrometry to identify all of the proteins in a complex mixture and quantify them at the same time. The problem is these approaches have poor precision. However, they've been extremely important in pushing basic science forward.

Slowly over time, people have been realizing that precise assays would be more beneficial to clinical research where large numbers of patients have a fair amount of intraindividual variability. Trying to discern differences between groups biologically, we would need precise assays.

So the field started to tend away from the shotgun experiments and to targeted experiments where we know what we are looking for. We are not trying to identify all the proteins. We know what we are looking for and now it's time to quantify it very precisely and accurately, ideally.

So many groups have attempted to improve the precision by looking at peak areas from chromatograms instead of trying to count the number of times, peptides are identified using shotgun approaches and the use of internal standards. These are isotope label; they are actually stable isotope, it’s not radioactive. But stable isotope

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labeled internal standard peptides to control for some of the variability of the mass spectrometric experiment which includes things like matrix effects and variable mass spectrometer performance overtime.

This approach, which is called isotope dilution mass spectrometry, which is the standard of care for small molecules in the clinical laboratory, was used to measure peptides in complex specimens and those peptides can either be naturally occurring in human samples or they could be generated by digesting all the proteins in a sample using trypsin or another protease.

Those peptides then behave as surrogates for the protein in the sample and by measuring those peptides, we can get a more precise measurement than the shotgun approaches. So for the past couple of years, people have used this isotope dilution approach, but haven't ever really looked at clinically useful proteins before in clinically useful matrices like human serum or plasma. So there has been a fair amount of work done leading up to what we have done.

Bob Barrett: So it doesn’t sound like it's a new idea. It sounds like scientists have been measuring proteins for a long time using mass spectrometry. How did your group’s studies differ from those previous experiments?

Dr. Andrew Hoofnagle: Some of the previous experiments really highlighted the fact that trypsin, which is the most common protease that we use in measuring proteins in complex matrices using mass spectrometry, the digestion using trypsin is very variable from day-to-day, and it's not completely understood why that’s true.

The steps that are involved in trypsin digestion include denaturation of all of the proteins. So removing all of the protein structure and turning them into linear polypeptides as much as possible, reducing all of the disulfide bonds using something like dithiothreitol or another reagent and then alkylating the cysteines that are present in the sample, again using something like iodoacetamide, although there are other reagents that can be used.

This denaturation step allows trypsin to attack the proteins and cut it as completely as possible. Unfortunately, from day-to-day, something is

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variable about the denaturation process or about the protein digestion.

So one thing that our group really focused on was how can we try to control for that day-to-day variability and there has been some really important papers showing from laboratory to laboratory that we can do a really good job of quantifying peptides very precisely between laboratories. But the moment that we introduce trypsin and start to digest those samples in different laboratories, we get different results across laboratories.

So we have day-to-day variability within laboratories and between laboratory variability, and our group was trying to focus on how to control for that variability.

And what we did was try to identify a denaturation protocol that was as robust as possible, and we found one using trifluoroethanol, which was a method that had been previously presented at a conference years ago.

In addition, we included a single calibrator, which was a native human serum sample that had properly folded proteins, so that those proteins would behave in a denaturation and trypsin digestion steps as close as possible to the patient samples that we were analyzing at the same time.

And it turned out that using that as the calibrator the native proteins rather than purified protein spiked into an appropriate matrix was much better at controlling for the day-to-day variability. Those proteins behaved much more like the proteins that we have naturally in our serum or plasma than purified standards could do.

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Bob Barrett: How did you pick the proteins that you measured?

Dr. Andrew Hoofnagle: We picked apolipoprotein A-I and apolipoprotein B, which are proteins that are already great proteins in the clinical laboratory. We use immunoassays to measure apoA-I and apoB all the time.

apoA-I is actually the structural protein for high-density lipoprotein which is the good cholesterol and apoB is the structural protein for LDL or the bad cholesterol. The two proteins are completely different. apoB is very, very large. It has many,

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many disulfide bonds; it has many glycosylation sites, such that 10% of the protein mass is actually carbohydrate. apoA-I is very simple, very small, it's less than a tenth of the size of apoB. It has no disulfide bonds, it has no glycosylation sites.

So they are extremely different proteins, very relevant to human biology already and very important in clinical medicine. So we decided to focus on these two very different proteins to see if we could measure them at the same time and provide some data regarding markers that people already knew in clinical care. They are very abundant proteins, which also made it attractive. We didn't need to enrich for the proteins at all. We were hoping to be able to digest plasma without any enrichment of the proteins from plasma and to be able to measure it directly.

Bob Barrett: Well, you mentioned that these proteins are present in blood at relatively high concentration. Could mass spectrometry be used for less prevalent blood proteins?

Dr. Andrew Hoofnagle: Absolutely, and there have been some important studies to date that show that using the antibodies rather than as primary reagents under detection of proteins, instead using those antibodies to purify either proteins or peptides from plasma or digested plasma could be used to lower the limits of detection or the sensitivity of the assay.

One problem using antibodies to purify intact proteins is that the autoantibodies that mask the epitopes in traditional immunoassays, those autoantibodies would also mask the epitopes of the proteins in our new mass spectrometric assay. They would bind the epitope and interfere with the binding of our reagent antibody.

However, if we digest all of the proteins in the sample into peptides, and then use an antibody to fish out the peptides that we're interested in analyzing, it's possible that we could actually go much lower and identify proteins that important in cancer biology as well as in other diseases. Our group has actually published an example of that of thyroglobulin and other investigators have published an analysis of troponin and other clinical samples as well.

So I think that we've made important steps towards measuring low abundance proteins using mass

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spectrometry. So we have really spanned high concentrations all the way down to the lowest concentrations that might be useful in clinical medicine.

Bob Barrett: So what else needs to be done before mass spectrometry can be used regularly to measure proteins in patient samples?

Dr. Andrew Hoofnagle: Well, I think the first thing that we need to do is to figure out how to make trypsin digestion as robust as possible and if it's not trypsin, we need a different protease.

So we need to understand how to make the denaturation step and the trypsin digestion step as robust as possible. How to make calibrators reliable to eliminate the day-to-day variability and the site-to-site variability and we need more and more laboratories to try to evaluate these methods to determine if this is an approach that could be transferred from laboratory to laboratory. It's sure to have huge benefits in terms of the standardization of protein measurements from lab to lab to direct translation of large clinical studies that use this type of assay in their clinical trials. The same exact assay would be used in the clinics, which is not true today with immunoassays.

The immunoassays that we use in the clinics are often very different from the assays that are used in large clinical studies that sort of defined the physiology of human beings. We don't have those assays available to us in the clinical laboratory. However, if we were able to translate the mass spec assays directly to clinical care, it would remove that problem altogether.

Bob Barrett: Well, it sounds like an achievable goal. What does the FDA have to say about all this?

Dr. Andrew Hoofnagle: That's a great question. The FDA is currently revisiting how they feel about laboratory developed tests in general. So mass spectrometric assays, both for small molecules and those that will be developed for proteins, are laboratory developed tests, meaning they've never had to be approved or cleared by the FDA previously.

So if the FDA decides that they're going to require FDA approval of laboratory developed tests, mass spec assays would fall into that category.

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Currently, there are very few mass spectrometers. I believe there is actually only one mass spectrometer that has ever been approved by the FDA as an instrument. So there isn't even an instrument that we as clinical laboratories could develop our assays for, and hopefully that would change in the near future.

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But currently, if the FDA decided to require approval of our mass spectrometric assays either of small molecules or large molecules, it would be a very complicated and long road.

Bob Barrett: Well, then what about insurance companies, will they pay for test that laboratories run using mass spectrometry?

Dr. Andrew Hoofnagle: I think the answer is complicated. Insurance companies as long as there is an analyte that is recognized as a useful analyte, they will pay for mass spectrometry to be used in the analysis of that analyte.

However, if these are brand-new biomarkers and proteomics has been trying to find novel biomarkers for a very long time, novel biomarkers are much more difficult to get paid for. That's going to be interesting over the next few years, but currently, if I were to measure apoA-I and apoB by mass spectrometry, I would be reimbursed at the same level as if I used an immunoassay. And the same can be said for thyroglobulin, for troponin, for the other assays that have preliminary results in the literature so far.

So I think that insurance companies will pay for current analytes. As new analytes get developed, they need to be verified in a large clinical study. They have to be shown to improve patient care and that's something that people have been working on for years for many of the biomarkers that have surfaced on the literature 5-10 years ago, but we still don't have great clinical studies to show that patient outcome is improved.

So I think that insurance companies will certainly pay to use mass spectrometry. I don't think that they are bothered by the technology, but the analytes themselves need to be verified in a large clinical study.

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Bob Barrett: And finally, let's look ahead. Where do you see mass spectrometry in the clinical lab in, say, 5-10 years?

Dr. Andrew Hoofnagle: Well, I guess I'm an optimist. Maybe that's inappropriate, but I'm hoping that mass spectrometry lives as a method of detection in automated analyzers in our clinical laboratories. Right now, we use things like spectrophotometry, ion-selective electrodes, things that have been around for very long time. And there's no reason that mass spectrometry can't become a novel method of detection in automated analyzers. Since I'm hoping in the next 5-10 years, we actually see an automated mass spectrometric clinical analyzer available for routine use in more and more laboratories around the country.

To address the question specifically of proteins, I again, am hopeful that in the next five years, we will make enough fundamental findings that we can use mass spectrometry to quantify proteins in a clinical laboratory on a regular basis.

So we will need things like automation and robotics and automated sample flow to allow that to happen on a large scale, but I think that more and more clinical laboratories are going to realize that measuring proteins by mass spectrometry can be affordable and is good for patient care.

So I'm optimistic over the next 5-10 years that we will see mass spectrometry become much more essential in the clinical laboratories as a method of detection both for small molecules as well as proteins.

Bob Barrett: Dr. Andrew Hoofnagle is a medical director of the clinical laboratories at the University of Washington in Seattle. He specializes in measuring concentrations of molecules using mass spectrometry in patient samples. He has been our guest in this podcast from *'Clinical Chemistry'*.

I am Bob Barrett. Thanks for listening.

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